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Morphological identification of species of the Nuneztovari Complex of *Anopheles* (Diptera: Culicidae) from an area affected by a Brazilian hydroelectric plant

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Abstract

The Nuneztovari Complex of *Anopheles* (Diptera: Culicidae) comprises four species: *An. nuneztovari* Gabaldon, *An. goeldii* Rozeboom & Gabaldon, *An. dunhami* Causey and *An. nuneztovari* species A. This study aimed to identify morphologically the species of the Nuneztovari Complex that occur in the area of the Belo Monte hydroelectric dam. The morphological identification of adult males and male genitalia (aedeagus and ventral claspette) was performed. A statistical analysis of the difference in aedeagal leaflet length was done using the Mann-Whitney test. Of the 38 male genitalia of specimens of the Nuneztovari Complex examined, 33 were identified as *An. goeldii*/*An. nuneztovari* A and five as *An. nuneztovari s.s*. A statistically significant difference in aedeagal leaflet length was detected between the species: the mean length was 1.23 μm for *An. goeldii*/*An. nuneztovari* A and 9.18 μm for *An. nuneztovari s.s*. This is the first record of *An. nuneztovari s.s.*in areas of environmental modification in the Brazilian Amazon. This study provides a measurement tool that can identify and differentiate species of the complex in the region, which can be applied to the other species of the complex as well to other anopheline species; thus, fostering the acquisition of information about the role of each species in malaria transmission.

Key words: *Anopheles*, similar species, *dunhami*, *goeldii,* malaria, male genitalia, *nuneztovari*

Introduction

Anopheles (*Nyssorhynchus*) *nuneztovari* was first described by Gabaldon (1940) when analyzing the genitalia of adult male specimens collected in San Carlos, Cojedes State, Venezuela (Faran 1980; Rubio-Palis 2000). The species is found from eastern Panama to northern South America and throughout the Amazon basin, occurring in Bolivia, Brazil, Colombia, Ecuador, Guyana, Peru and Venezuela (Sinka *et al.* 2010). In Brazil, it occurs in states in the Amazon region (Kitzmiller *et al*. 1973; Faran 1980; Consoli & Oliveira 1994; Manguin *et al*. 2008).

Studies on behavior, morphology, cytogenetics and molecular taxonomy indicated that *An. nuneztovari* is a species complex, which exhibits a wide variety of bionomical characteristics in different localities (Kitzmiller *et al*. 1973; Faran 1979). In the Brazilian Amazon, *An. nuneztovari* was considered a local malaria vector (Galardo *et al.* 2007) with a preferential zoophilic, exophilic and crepuscular biting pattern (Elliot 1968, 1972; Panday 1977; Tadei & Correia 1982; Tadei & Thatcher 2000). In Venezuela and Colombia, *An. nuneztovari* is a primary vector (Lounibos & Conn 2000; Altamiranda-Saavedra *et al.* 2017; Naranjo-Díaz *et al.* 2018) where it exhibits an endophilic habit with peak biting in late hours, near midnight (Elliot 1968; Rubio-Palis & Curtis 1992). In Venezuela there is a certain balance between the endophilic and exophilic habits, but in Colombia both endophily and anthropophily are evident (Fajardo & Alzate 1987; Rubio-Palis & Curtis 1992).

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After the initial description of *An. nuneztovari*, Rozeboom & Gabaldon (1941) described a similar species collected in the Fordlândia District, Aveiro municipality, state of Pará, Brazil, named *An. goeldii*, distinguishable from known species by the male genitalia (Faran 1980). A similar species from Tefé, Amazonas State, Brazil, *An. dunhami*, was described by Causey (1945), egg, larval and adult characteristics of which are similar to those of *An. goeldii* but whose male genitalia are distinct from those of *An. nuneztovari*.

A cytogenetic study showed that specimens of *An. nuneztovari* from Colombia and Venezuela were distinguished from Brazilian specimens by an inversion on the X chromosome (Kitzmiller *et al*. 1973). Faran (1980) did not find significant morphological differences among specimens from Brazil, Guyana and Venezuela, and considered all the specimens to be *An. nuneztovari*. However, due to the behavioral and chromosomal differences reported in previous studies, it was hypothesized that *An. goeldii* is a valid species. Gabaldon (1981) detected morphological differences in the male genitalia and the fourth-instar larva, corroborating the hypothesis. Conn (1990) and Conn *et al*. (1993) conducted cytogenetic analyses on specimens of *An. nuneztovari* from different geographic areas and recognized three distinct cytotypes (A, B and C) correlated with populations in Brazil (A), Venezuela (B) and Colombia (B/C). Peyton (1993), after morphological analysis of the male genitalia, validated *An. dunhami* as distinct from *An. nuneztovari*. Scarpassa *et al*. (1996) detected significant differences between populations of Brazilian and Colombian *An. nuneztovari* based on alloenzyme analysis, suggesting reproductive isolation between them. Subsequently, a mitochondrial DNA analysis of populations from the Brazilian Amazon and Colombia suggested that these anophelines were different species (Scarpassa *et al*. 1999; Scarpassa & Tadei 2000). Bergo *et al.* (2007) proposed that *An. goeldii* is a valid species after a morphological analysis of the aedeagus of males collected in the Brazilian Amazon (Amapá), which was previously identified as *An. nuneztovari*. Analysis of the nuclear *white* gene in populations of the Nuneztovari Complex from Bolivia, Brazil, Colombia, Suriname and Venezuela detected five different genetic lineages, three of which occur in the Brazilian Amazon (Mirabello & Conn 2008). Based on morphological characteristics of the apex of the male aedeagus, Calado *et al*. (2008) found that *An. goeldii* is similar to specimens of *An. nuneztovari* A collected in municipalities from Pará, Amapá and Amazonas states, but is distinct from *An. nuneztovari s.s* (previously cytotype B/C).

Anopheles dunhami had already been recorded from Brazilian Amazon states: Amazonas, the municipalities of Tefé (Causey 1945), Tabatinga (Lounibos *et al.* 1998), Coari (Trindade & Scarpassa 2002) and Parintins (Scarpassa & Conn 2011); Pará, the municipality of Itaituba (Scarpassa & Conn 2011); and Rondônia, the municipality of São Miguel (Scarpassa & Conn 2011). The species was first recorded in the Amazon Department of Colombia by Ruiz *et al*. (2010), and in the Loreto Department of Peru by Moreno *et al*. (2015). Prussing *et al*. (2018) reported for the first time this species (as *Nyssorhynchus dunhami*) naturally infected by *Plasmodium vivax* and *P. falciparum*, which provided evidence of its role in malaria transmission, at least in eight localities in peri-Iquitos, Peru, where specimens were collected.

Morphological studies of male genitalia and molecular approaches using the internal transcribed spacer (ITS2) region of ribosomal DNA, the cytochrome c oxidase subunit I (*COI*) gene of mitochondrial DNAand the *white* gene of nuclear DNA from specimens collected in Amazonian states have shown that *An. nuneztovari* and *An. goeldii* are distinct species with different geographical distributions, with *An. goeldii* and *An. nuneztovari* A occurring in the Brazilian Amazon and *An. nuneztovari* in Colombia and Venezuela (Calado *et al*. 2008).

Scarpassa & Conn (2011) analyzed the *COI* gene and concluded that *An. nuneztovari* is a complex of at least two morphologically similar species, with *An. nuneztovari* occurring in Bolivia, Colombia and Venezuela and *An. goeldii* and *An. nuneztovari* A occurring in the Amazon basin. *Anopheles dunhami* was mentioned only as a related species. Based on phylogenetic and morphological analyses, Foster *et al*. (2013) suggested the inclusion of *An. dunhami* in the Nuneztovari Complex. Subsequently, Sant'Ana *et al*. (2015) re-described *An. goeldii*, concluding that it is a valid species differentiated from *A. nuneztovari* by characteristics of the fourth-instar larva, adult females and males, and the male genitalia. Based on all of these studies, *An. nuneztovari* is a complex of four species: *An. dunhami*, *An. goeldii*, *An. nuneztovari* and *An. nuneztovari* A.

Thus, this study aimed to identify species of the Nuneztovari Complex collected in the vicinity of the Belo Monte Hydroelectric Power Plant (Belo Monte HPP) based on morphological characteristics of the male genitalia, and to define other possible means to differentiate the species.

Materials and methods

Specimens included anopheline larvae and pupae collected in aquatic habitats in the municipalities of Altamira, Bonanza Farm (03° 10′ 21.9″ S, 52° 08′ 51.7″ W) and Vitória do Xingu (Dam 29 and Transcatitu Road, 03° 17′ 34.1″ S, 51° 54′ 12.0″ W), both located in the area of the Belo Monte HPP, Pará, Brazil. The local habitats searched were permanent lakes with clean water fully exposed to sunlight. Habitats located in Bonanza Farm and Transcatitu Road had emergent vegetation and those at Dam 29 had floating vegetation (macrophytes).

The larvae and pupae were collected using entomological dippers, placed in tubes containing water from the habitat and associated with the location and time of collection, and transported to the field laboratory. The specimens were then kept in plastic containers (40 × 25 cm) containing tap water and were fed twice daily with crushed fish food (TetraMin®). Maintenance and cleaning of the plastic containers was performed daily by the removal of excess debris and the partial replacement of water with clean water.

Emerged adults were identified to species using the morphological identification keys of Gorham *et al*. (1967), Consoli & Oliveira (1994) and Forattini (2002). Males identified as members of the Nuneztovari Complex had their genitalia dissected, the aedeagus separated from the rest of the genitalia and assembled according to the protocol provided by the Entomology Laboratory of the Faculty of Public Health of São Paulo, with modifications. The dissected genitalia were placed in 20% KOH for approximately 5 h. The KOH solution was replaced by 20% acetic acid, which was removed after 10 min. Subsequently, 1% Acid Fuchsin solution was added for approximately 20 min or until the genitalia were rose-colored. The specimens were then dehydrated 10 min each through a series of 80, 90, 95% and absolute ethanol. A second clearing was undertaken in Xylol for 30 min. The specimens were mounted in Canada balsam on microscope slides and allowed to dry at room temperature.

Genitalia were observed under an optical microscope at magnifications of 100 and 400x. The identification of species was based on characteristics of the apex of the aedeagus and the ventral claspette. After morphological identification, the aedeagal subapical leaflets were examined under 1000x magnification and the distance between the lower base to the apex of each leaflet was measured using the AxioVision Microscopy® program (Fig. 1).

To determine whether the difference in the length of the leaflets was statistically significant between the species, the Mann-Whitney test was performed because the measurements were independent samples. For verification of sample normality, the D'Agostino and D'Agostino-Pearson tests were used. All tests were performed in the Biostat 5.3® program (Ayres *et al*. 2007). The level of significance was $\alpha = 0.05$, where differences were considered significant when $p < 0.05$.

Results

Forty-six adult males reared from larvae were identified morphologically as members of the Nuneztovari Complex. The genitalia were dissected from 38 of the males, and 33 were identified as *An. goeldii/An. nuneztovari* A based on the subapical leaflets of the aedeagus being either absent or small (observation was difficult due to the apex of the aedeagus having a membranous aspect) (Fig. 2A, B). The remaining five genitalia were identified as *An. nuneztovari* based on the apex of the aedeagus being large with well-developed subapical leaflets, markedly larger than in *An. goeldii/An. nuneztovari* A (Fig. 2C–E).

In addition to the aedeagus, the ventral claspette of the specimens was examined and found to exhibit typical characteristics for each species: *An. goeldii/An. nuneztovari* A – refringent structure not racket form and without evident lateral arms (Fig. 2F: 1), pre-apical plate larger than in *An. nuneztovari* (Fig. 2F: 2) and shallow median groove with rounded outer corners (Fig. 2F: 3). *An. nuneztovari* – refractive racket structure with evident lateral arms (Fig. 2G: 1), small pre-apical plaque (Fig. 2G: 2) and a medium shallow groove in the shape of the letter V with sharp outer corners (Fig. 2G: 3). None of the specimens examined exhibited the morphological characteristics of the male genitalia of *An. dunhami*.

The aedeagal subapical leaflets of *An. goeldii/An. nuneztovari* A ranged from absent to 5.96 μm in length, whereas in *An. nuneztovari* they ranged from 6.97 to 14.81 μm (Table 1). The statistical analysis revealed a significant difference in the length of the subapical leaflets between the species ($p = 0.0015$) (Fig. 3).

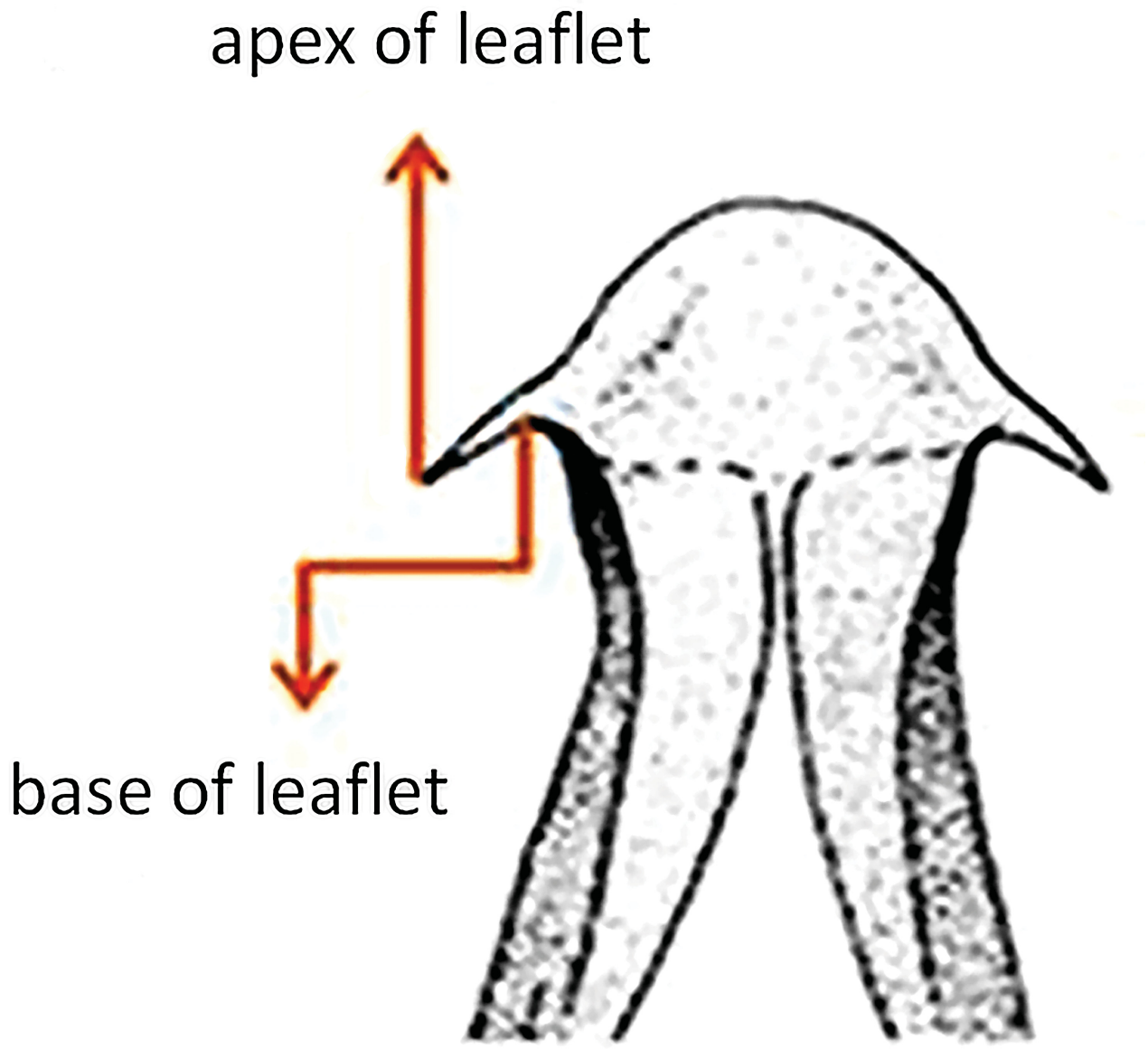

FIGURE 1. Drawing of the aedeagus showing the base and apex of the aedeagal subapical leaflets.

TABLE 1. Length of the aedeagal subapical leaflets of *An. goeldii/An. nuneztovari* A and *An. nuneztovari* from the area of the Belo Monte HPP. Measurements in μm.

Species	n	Mean	Minimum	Maximum	Standard deviation
An. goeldii/An. nuneztovari A	33	1.2296	0.0	5.96	2.0473
An. nuneztovari s.s.	05	9.1825	6.78	14.81	3.8018

Discussion

Studies on anopheline mosquitoes, vectors of malaria, are needed to generate knowledge that can guide vector control actions. Aspects of the bionomics of these mosquitoes, including density and diversity, can be altered by human interventions, such as the construction of hydroelectric plants. This type of activity causes environmental changes, such as deforestation and the formation of channels and lakes, which can become new habitats for the immature stages of these mosquitoes. Thus, the abundance of members of the Nuneztovari Complex commonly

increases in these areas (Forattini 2002). The relationship between the prevalence of the Nuneztovari Complex and the construction of hydroelectric plants has already been demonstrated in other studies, such as in the areas of influence of the Balbina (Amazonas) and Tucuruí (Pará) hydroelectric plants. The Nuneztovari Complex comprised 94% of the anopheline mosquitoes collected in Balbina, and 90% in Tucuruí (Quintero *et al*. 1996; Tadei *et al*. 1998).

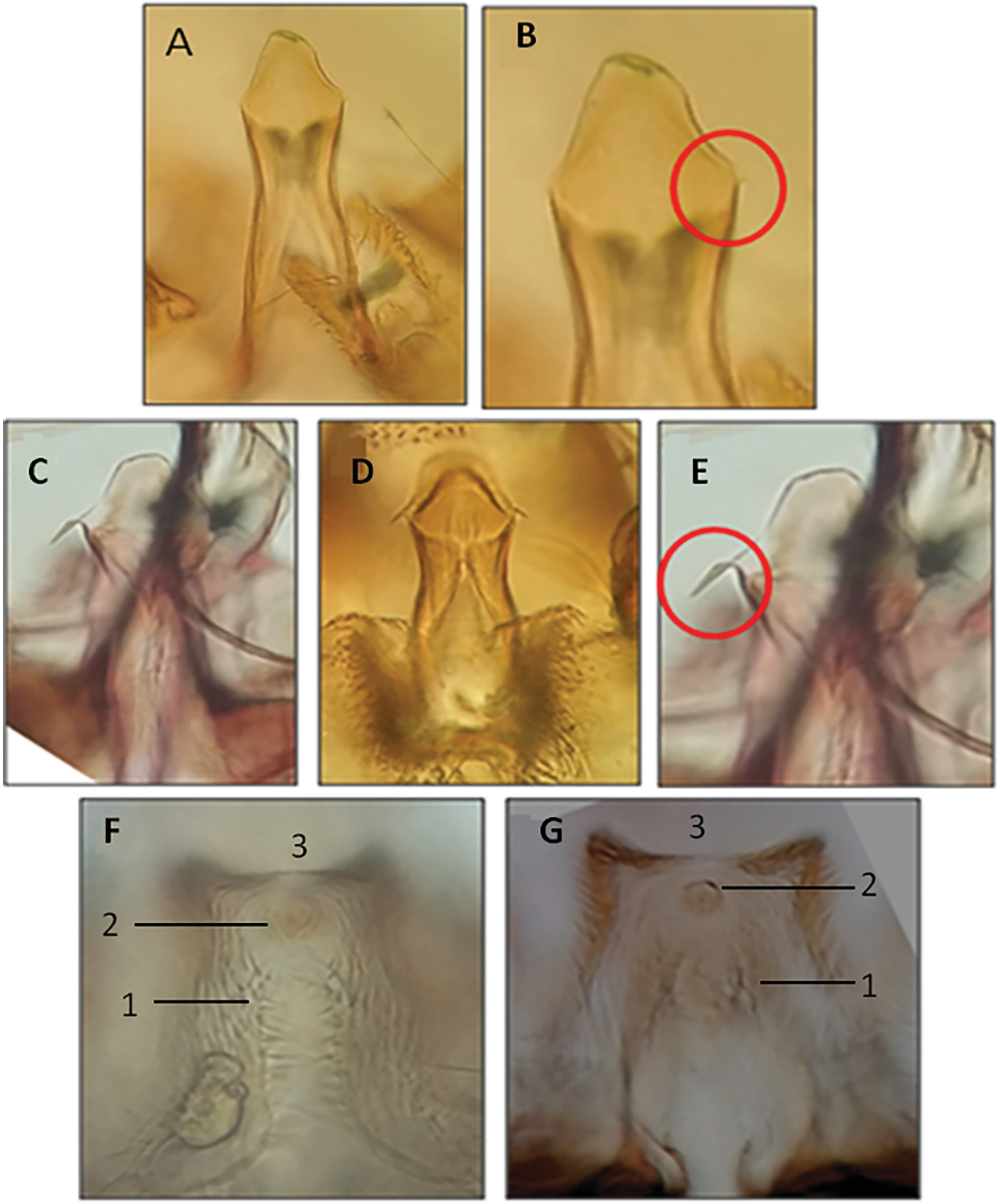

FIGURE 2. A–F, Aedeagal morphology: A–C, *An. goeldii/An. nuneztovari* A; D–F, *An. nuneztovari*. The subapical leaflets are enclosed in red circles. G,H, Ventral claspette: G, *An. goeldii/An. nuneztovari* A; H, *An. nuneztovari.*

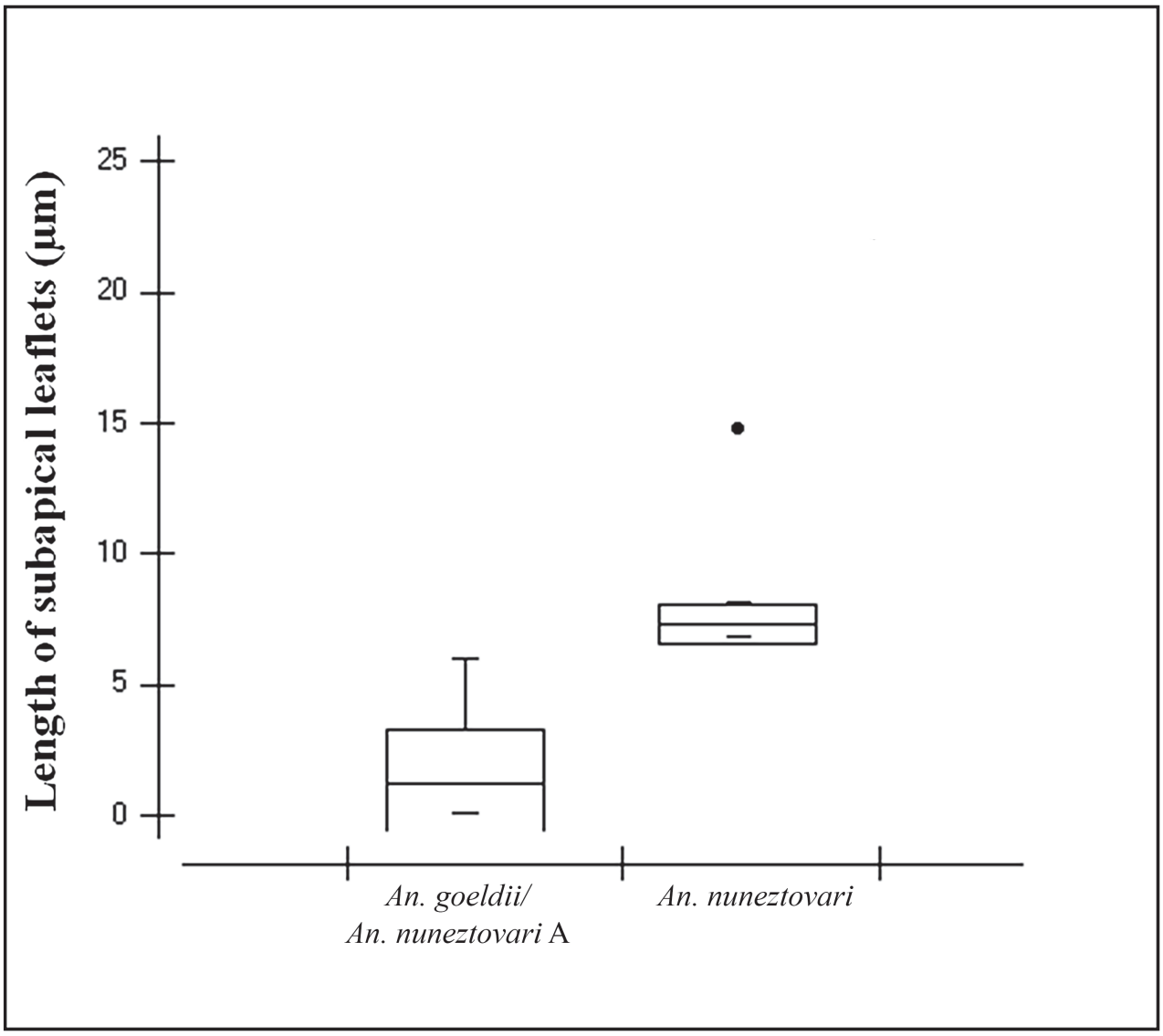

FIGURE 3. Difference in length of the subapical leaflets between *An. goeldii/An. nuneztovari* A and *An. nuneztovari* collected in the area of the Belo Monte HPP.

In 2012, construction of the Belo Monte HPP started and environmental changes were not yet detectable and visible, and *An. darlingi* was the predominant species in all areas around the plant, representing 70% of all specimens collected, whereas the Nuneztovari Complex represented only 10% of the specimens (Póvoa *et al*. 2012). In 2015 and 2016, during the final phase of construction, members of the Nuneztovari Complex became the most numerous, 67% of all collected specimens, and *An. darlingi* represented only 3% (Póvoa *et al.* 2015, 2016).

Because the Nuneztovari Complex comprises four species and not all have been incriminated as malaria vectors, it is very important to distinguish them in areas of malaria transmission. To that end, there is a need for methods that can be performed in areas where the only available tool is a microscope, which is the case in Amazonian states and municipalities.

The morphological examination of male genitalia as a method to identify members of species complexes is inexpensive and simple to perform. Causey *et al*. (1946) constructed an identification key for anophelines of the Brazilian Amazon and Brazilian northeastern regions based on the male genitalia. The morphological studies of male genitalia enabled species of the Nuneztovari Complex to be differentiated based mainly on variation of the apex of the aedeagus (Gabaldon 1981; Savage 1986; Peyton 1993; Bergo *et al.* 2007; Calado *et al.* 2008; Sant'Ana *et al.* 2015). The revalidation of *An. goeldii* was based on such morphological characteristics (Bergo *et al*. 2007). Studies on male genitalia in association with molecular analyses have led to the distinction of *An. nuneztovari* and *An. goeldii*, and have revealed morphological similarity between *An. goeldii* and *An. nuneztovari* A (Calado *et al.*

2008), resulting in the inclusion of *An. dunhami* in the Nuneztovari Complex (Foster *et al.* 2013) and the re-description of *An. goeldii* (Sant'Ana *et al*. 2015).

The specimens collected and identified in the present study belong to two or three of the four species of the Nuneztovari Complex: *An. goeldii/An. nuneztovari* A and *An. nuneztovari*. One difficulty faced in the morphological analysis of the male genitalia was the subjectivity of characteristics of the aedeagal subapical leaflets. Thus, numerical differences in the measurements that can define each species as shown here, i.e. at least for two of the four species, are of a great importance. As members of the Nuneztovari Complex had already been incriminated as malaria vectors in the Brazilian Amazon (Galardo *et al*. 2007), it is very important investigate it in the study area in order to warn the local health authorities of their possible role in malaria transmission.

The length of the aedeagal subapical leaflets of the specimens of *An. nuneztovari.* and *An. goeldii/An. nuneztovari* A was measured, and a statistically significant difference was observed, reinforcing the idea that the subapical leaflets are structures that can be used to distinguish species of the Nuneztovari Complex. Even so, it is considered prudent to subject the specimens to molecular analysis to confirm the results because Scarpassa *et al*. (2016) have recently reported the presence of three different genetic strains of the Nuneztovari Complex in the Amazon region, two corresponding to *An. goeldii* and one that may be a new species genetically different from *An. dunhami* and *An. nuneztovari*. This lineage was detected in Tucuruí, Pará, relatively close (405 km) from the area of the present study.

Here, the specimens identified as *An. goeldii/An. nuneztovari* A showed slight variation in the length of the subapical leaflets, which were absent or almost imperceptible (up to 5.96 μm), whereas in *An. nuneztovari* the length ranged from 6.78 to 14.81 μm. The criterion for identification was based on the study conducted by Sant'Ana *et al.* (2015), which described the leaflets of *An. goeldii* as absent or small, membranous and difficult to see. However, because the subapical leaflets can be folded, and thus can be difficult or impossible to discern, Sant'Ana *et al.* also examined the ventral claspette. Based on this, the specimens were also identified as either *An. goeldii/An. nuneztovari* A or *An. nuneztovari.*

The literature records the occurrence of *An. goeldii/An. nuneztovari* A in different Brazilian Amazon states (Rozeboom & Gabaldon, 1941; Bergo *et al*. 2007; Mirabello & Conn 2008; Calado *et al.* 2008; Scarpassa *et al*. 2016), including Itaituba and Altamira municipalities in Pará State. Thus, the occurrence of this species in the study area represents the confirmation of previous records. In contrast, no records of *An. nuneztovari* were found, which indicates that this is the first record in this region. Calado *et al.* (2008) reported the presence of this species in Colombia and Venezuela, and Scarpassa & Conn (2011) recorded the species in Bolivia, Colombia and Venezuela.

Based on molecular analysis of specimens collected in the area of the Belo Monte HPP in 2014 (data not published), two species of the complex, *An. goeldii* and *An. nuneztovari*, were identified, corroborating the results of the present study. Therefore, it is recommended that molecular analyses should be conducted in future studies for species confirmation.

Anopheles dunhami was not found in our study, but it has been recorded from states in Brazilian Amazonia (Causey 1945; Lounibos *et al*. 1998; Trindade & Scarpassa 2002; Scarpassa & Conn 2011). In Colombia, it was first recorded by Ruiz *et al.* (2010). Considering that this species has already been found in the municipality of Itaituba of Pará State, which is only 489 km from Altamira, we believe that the non-encounter of this species is probably due to the sample size. Thus, a larger sample of specimens from the area of the Belo Monte HPP needs to be analyzed, mainly because Prussing *et al*. (2018) found *An. dunhami* naturally infected by both *P. falciparum* and *P. vivax* in areas of Iquitos, Peru, and advocated its role in malaria transmission and the need for precise identification of this species as it has been misidentified as other species of *Anopheles*.

The increase in populations of the Nuneztovari Complex and the presence of malarial vectors of this complex in areas of environmental transformation, such as the construction of hydroelectric plants (Tadei *et al*. 1998; Tadei & Thatcher 2000; Forattini 2002), are risk factors for the transmission of malaria. Members of the Nuneztovari Complex have already been incriminated as local malaria vectors in areas of the Brazilian Amazon (Galardo *et al*. 2007), and *An. nuneztovari* is considered a vector in areas of Colombia and Venezuela (Faran 1980; Rubio-Palis 2000). Thus, it is important to identify the individual species of the complex in the study area to assess the risk of their involvement in malaria transmission and consequently to help the local health authorities to establish proper control strategies.

The morphological examination of male genitalia, which is an efficient method for many reasons, and can be performed by trained technicians, is suitable for use in the field because it does not require a large infrastructure, a

controlled environment and is inexpensive. Furthermore, considering that in many Brazilian areas of malaria transmission the entomology groups have facilities which limit them to methods of morphological identification, this study provides another useful tool for the identification of two species of the Nuneztovari Complex.

Conclusions

The results of this study show that the morphological examination of the male genitalia is a viable method for identification of species of the Nuneztovari Complex. Two or possibly three species of this complex were found in the study area, *An. goeldii/An. nuneztovari* A and *An. nuneztovari*. Measurements of the length of the subapical leaflets of the aedeagus can be used as a tool to differentiate these species. In addition, this was the first record of *An. nuneztovari* in an area where a hydroelectric plant is under construction in the Brazilian Amazon.

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